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Impaired Expansion of Mouse B Cell Progenitors Lacking Btk

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Summary

Mutations in the gene encoding the protein tyrosine kinase Btk are associated with the human B cell immunodeficiency X-linked agammaglobulinemia (XLA). In the mouse, a point mutation in the Btk pleckstrin homology domain segregates with a milder X-linked immunodeficiency (*xid*). To assess the importance of Btk function in murine lymphopoiesis, we generated multiple embryonic stem cell clones bearing a targeted disruption of the *btk* gene and examined their potential to produce lymphocytes in both C57BL/6 and *RAG2*$^{-/-}$ host chimeric animals. These mice provide a complementary set of in vivo competition assays that formally establish the genetic basis for the *xid* phenotype. Although the *null* mutation yields a phenotype quite similar to that of *xid*, it also compromises expansion of B cell precursors. Our results suggest that the murine and human consequences of Btk deficiency differ only quantitatively, and represent the same disease process.

Introduction

B lymphocytes develop from bone marrow progenitors via an ordered series of differentiative steps characterized by immunoglobulin gene rearrangement, proliferation, and the expression of numerous gene products (Osmond, 1986; Hardy et al., 1989, 1991; Li et al., 1993; Melchers et al., 1994). Mutations in the gene encoding the Bruton tyrosine kinase (Btk; Rawlings and Witte, 1994; Conley et al., 1994; Smith et al., 1994a) are responsible for X-linked agammaglobulinemia (XLA), a severe primary immunodeficiency of boys characterized by the virtually complete absence of circulating B lymphocytes. A mutation in the mouse *btk* gene is linked genetically with the relatively mild X-linked immunodeficiency (*xid*) of CBA/N animals (Rawlings et al., 1993; Thomas et al., 1993).

Btk is expressed widely throughout hematopoietic cell lineages, including nearly all stages of B cell development, but is found at negligible levels in T cells and is specifically down-regulated in plasma cells (Tsukada et al., 1993; de Weers et al., 1993; Smith et al., 1994b). The *BTK* locus, comprising 18 coding exons (Ohta et al., 1994) and mapping to Xq22 in the human (Vetrie et al., 1993; Tsukada et al., 1993), encodes a 659 aa protein tyrosine kinase (PTK) homologous to Tec II (Mano et al., 1993), Bmx (Tamagnone et al., 1994), Txk (Haire et al., 1994), Itk (Yamada et al., 1993; Siliciano et al., 1992), and Dsrc 28C (Gregory et al., 1987). This subfamily of lineage-restricted nonreceptor PTKs is reminiscent of, but distinct from, the c-*src* family of PTKs (reviewed by Perlmutter et al., 1993; Bolen, 1993). The src family kinases participate in numerous signaling pathways as components of the signaling machinery linking membrane-associated receptors to the nucleus.

Btk, like the src family PTKs, contains a unique N-terminal region, single SH3 and SH2 domains, and a kinase domain capable of both auto- and *trans*-phosphorylation. SH3 domains have been shown to interact with proteins containing a short proline-rich motif (Koch et al., 1991; Cicchetti et al., 1994; Ren et al., 1993), while SH2 domains interact with motifs containing phosphorylated tyrosine residues (Malek and Desiderio, 1993; Mayer et al., 1991; Pawson and Gish, 1992). The kinase domain of Btk possesses the conserved autophosphorylation site of src family kinases, but lacks the C-terminal regulatory tyrosine residue. Btk also lacks the N-terminal glycine residue, which serves as a myristylation site in src family PTKs.

The BTK N-terminal domain is unique, highly basic, and contains both a pleckstrin homology (PH) domain and two proline-rich motifs. PH domains, roughly 100 aa residues in length, are comprised of two antiparallel β sheets and a C-terminal α helix (Yoon et al., 1994; Ferguson et al., 1994; Macias et al., 1994). The PH domains present in the β adrenergic receptor kinase (βARK) and phospholipase Cγ have both been demonstrated to mediate binding to the βγ dimer of heterotrimeric G proteins that are liberated from the GTP-bound α subunit following receptor activation (Koch et al., 1991; Touhara et al., 1994). Several PH domains have been shown to bind the membrane lipid phosphatidylinositol-4,5-bisphosphate as well, implying a role in membrane localization (Harlan et al., 1994). The PH domain of Btk is comprised of 137 residues divided into six interspersed subdomains (Musacchio et al., 1993); a single tryptophan (W-124) in subdomain 6 is conserved among all known PH domains (Shaw, 1993). Despite relatively low overall sequence similarity with the PH domain of βARK (8%), the PH domain of Btk binds βγ in vivo and in vitro (Tsukada et al., 1994), with subdomains 5 and 6 both necessary and sufficient for the interaction. In addition, the Btk PH domain binds PKC β1, β2, ε, and ζ isoforms in vitro, and β1 in vivo in mast cells and B lymphocytes (Yao et al., 1994), leading to phosphorylation of Btk and

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down-regulation of its kinase activity. Mutation of the highly conserved arginine at position 28 in subdomain 2 has been observed in some patients with XLA (de Weers et al., 1994a; Ohta et al., 1994) and is associated with the *xid* phenotype in the mouse (Rawlings et al., 1993). The murine R28C mutation reduces PKC binding 3- to 5-fold (Yao et al., 1994). In distinct contrast, replacement of glutamine at position 41 with lysine activates the kinase: this mutant exhibits increased membrane targeting and phosphorylation on tyrosine, and its expression drives the growth of fibroblasts in soft agar and confers interleukin-5 (IL-5) independence upon a B lineage cell line (Li et al., 1995). In mast cells, FcεRI cross-linking induces activation and tyrosine phosphorylation of Btk, and measurable membrane translocation as well (Kawakami et al., 1994); similar activation occurs in B cells via surface immunoglobulin receptor ligation (Aoki, 1994; Saouaf et al., 1994; de Weers et al., 1994b).

The CBA/N mouse (*xid*) has served as a model of mild X-linked B cell–intrinsic immunodeficiency for over 20 years (Amsbaugh et al., 1972; Scher et al., 1973; reviewed by Wicker and Scher, 1986; Scher, 1982). Normal numbers of pro-B, pre-B, and surface immunoglobulin-positive (Ig^+) B cells are present in the bone marrow (Reid and Osmond, 1985; Kincade et al., 1982), and Abelson leukemia virus targets are present in normal numbers (Karagogeos et al., 1986; Osman et al., 1992) in these mice. Peripheral B cell numbers, however, are significantly reduced (30% of normal), and exhibit an immature IgM^{bright}, IgD^{dull}, and class II major histocompatibility complex–dull (MHC^{dull}) phenotype. These animals do not produce antibody when challenged with polysaccharide thymic-independent type 2 (TI-2) antigens (Wicker and Scher, 1986), and both IgM and IgG3 isotype levels are reduced (Perlmutter et al., 1979). In addition, B cell responses are either impaired or absent to a number of mitogenic stimuli, including lipopolysaccharide (Scher et al., 1975), surface immunoglobulin cross-linking (Sieckman et al., 1978), IL-5 (Hitoshi et al., 1993), IL-10 (Go et al., 1990), CD38 (Harada et al., 1993; Santos-Argumendo et al., 1995), and CD40 ligand (Hasbold and Klaus, 1994). B cells in female mice heterozygous for the *xid* mutation generally express the wild-type X, and this skewing becomes nearly absolute in mice over 3 months of age (Forrester et al., 1987; Nahm et al., 1983).

XLA was the first immunodeficiency to be described (Bruton, 1952), although its identification as a primary B cell immunodeficiency was more recent (Cooper et al., 1971; Pearl et al., 1978; Conley, 1985; Tedder et al., 1985; Campana et al., 1990). In XLA patients, $CD19^+/CD34^+$ early pre-B cells are present in the bone marrow in normal numbers, with no skewing of X inactivation in female carriers (Conley et al., 1994). Many XLA patients, however, have reduced numbers of cytoplasmic μ^+/sIg^- pre-B II cells (Campana et al., 1990), and in all patients studied the proliferative activity of cells at this stage appears consistently compromised. The phenotype of the few B cells that reach the periphery (less than 1% of normal numbers) is informative: these appear immature by virtue of the same surface-marker expression pattern that distinguishes *xid* B cells (Conley, 1985).

Meaningful comparison of the phenotypes seen in XLA and *xid* is complicated by an incomplete understanding of the consequences of the murine R28C mutation. To address this issue, we have generated multiple embryonic stem cell (ES) clones bearing a targeted disruption of the murine *btk* gene and analyzed their ability to produce lymphocytes in somatic cell chimeras created by the injection of these Btk^- ES cells into both wild-type and $RAG2^{-/-}$ blastocysts. Our results provide formal proof that mutations in the *btk* gene are responsible for the *xid* phenotype, and suggest that only quantitative variation distinguishes between the phenotypes imposed by Btk^- mutations in humans and mice.

Results

Disruption of the *btk* Gene in ES Cells and Generation of Somatic Cell Chimeric Mice

Several electroporations of disruption vector BTKN.1 (Figure 1A) into two ES cell lines derived from the 129 mouse strain yielded multiple disrupted clones. Of 360 D3J8 ES clones from two electroporations, 6 (1/60) were scored as containing homologous integrants via genomic blotting (Figure 1B); 315 REK2 clones were expanded from a single electroporation and 27 of 217 (1/8) analyzed clones contained the desired integration. The disruption eliminated exons 3 and 4, encoding all of PH subdomain 5, and most of subdomain 6 (including W124, the single most-conserved residue among known PH domains). Splicing from coding exons 2 to 5, if this occurs, would result in a frameshift mutation producing a stop in the first codon of exon 5. Reverse transcriptase–mediated polymerase chain reaction (RT-PCR) of sorted splenic B cells from wild-type (129) and $RAG2^{-/-}/Btk^-$ chimeric mice (Figure 1C) revealed that this splicing event occurs with a high degree of fidelity. A single ES-derived amplification product, consistent with splicing of exons 2 and 5 (Figure 1C, left), resulted when primers from exons 2 and 12/13 were used, suggesting that splicing from exon 2 to other downstream exons occurs rarely, if at all. To examine the specificity of the mutant splicing event, both wild-type and mutant fragments generated from a second amplification strategy using primers from exon 1 and 5/6 (Figure 1C, right) were isolated, cloned, and sequenced. We documented normal splicing for 4 of 4 wild-type clones, and precise exon 2-to-5 splicing for 4 of 4 mutant clones (data not shown). There is no evidence that any truncated form of Btk is produced naturally from initiation at either of two potential translation sites downstream of the deleted exons. In addition, the persistence of the known translational start site in exon 1 should render reinitiation quite ineffective, even if such occurs, since the translational efficiency of downstream reading frames in polycistronic messages is reduced 40- to 300-fold in mammalian cells (Kaufman et al., 1987). Hence, this disruption almost certainly represents a true *null* allele.

Because both of our ES lines are genetically male, disruption of a single allele is sufficient to create a *null* genotype. Given the need for only a single round of selection, the rapidity with which results might be obtained, and the

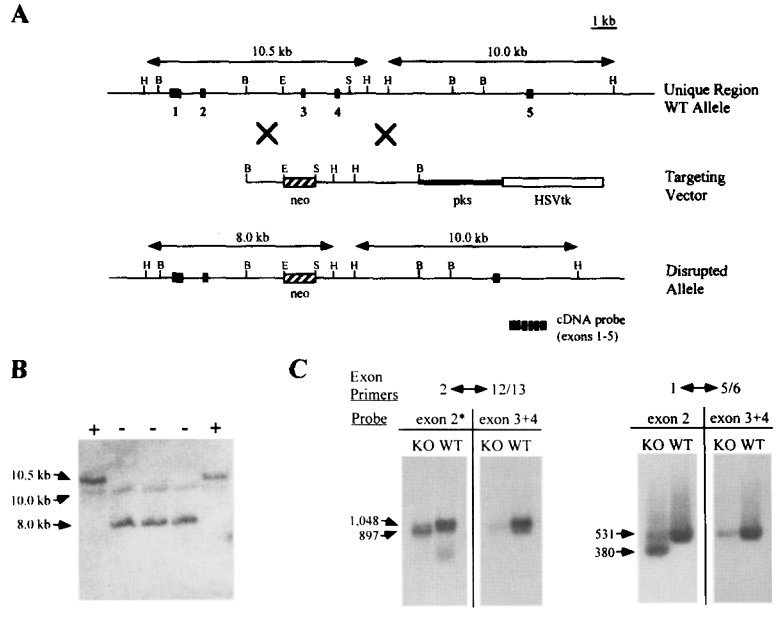

Figure 1. Targeted Disruption of the Murine *btk* Gene

(A) A partial restriction map of a 20 kb 129 mouse genomic clone including *btk* coding exons 1–5 (top), targeting vector BTKN.1 (middle), and predicted integration via homologous recombination (bottom). Sizes indicated are those resulting from HindIII digests, and contain coding exons 1–4 in the wild-type allele (10.5 kb), exons 1–2 in the disrupted allele (8.0 kb), and coding exon 5 (10 kb) in both.

(B) Southern blot of Hind III digests of genomic DNA from three disrupted ES clones (minus) flanked by two wild-type ES DNA samples (plus). A cDNA probe comprising *btk* coding exons 1–5 is completely external to the disruption vector and scores homologous integrational events (see [A]). Note that the lighter intensity band at 10.0 kb, *representing exon 5*, remains unchanged in the disrupted allele, while the 10.5 kb exon 1–5 band is shifted to 8.0 kb by the homologous event deleting exons 3 and 4. The presence of single integrations and loss of exons 3 and 4 in disrupted clones were documented via additional probing strategies (data not shown).

(C) RT–PCR of *btk* transcripts from sorted splenic B cells. Peripheral B cells (1×10^5) ($B220^+/CD3^-$) from a 129 control (WT) and a 4-month-old $RAG2^{-/-}$/Btk^- chimeric mouse (KO) (derived from REK-2 Btk^- clone 5/30) were isolated by FACS. Total nucleic acid preparations were used for first-strand cDNA synthesis with primers corresponding to the thirteenth (left) or sixth (right) *btk* coding exons. Amplification with a 5′ primer corresponding to the start of coding exon 2 and a 3′ primer spanning the junction between exons 12 and 13 generates predicted fragments of 1048 bp from wild-type transcripts and 897 bp from mutant transcripts lacking exons 3 and 4 (left), revealed via Southern blotting with an oligomeric probe (2*) corresponding to the 3′ end of exon 2. Probing with a pool of exon 3– and exon 4–derived oligomers demonstrates minor contamination of the chimeric B cells with $RAG2^{-/-}$-derived splenic monocytes. To address the fidelity of the predicted splicing from exons 2–5 in mutant-derived transcripts, first-strand cDNA was amplified with a 5′ primer corresponding to the start of coding exon 1 and a 3′ primer spanning exons 5 and 6. Fragments consistent with predicted sizes (531 bp for wild-type, 380 bp for mutant) were identified (right). Again, a minor wild-type transcript contamination from $RAG2^{-/-}$-derived cells was observed following probing with the combination of exon 3 and 4 oligomers. Mutant and wild-type PCR products were cloned and sequenced, revealing precise splicing from *btk* exons 2–5 (thereby introducing a termination codon) in mutant products (data not shown).

possibility of the detection of subtle competitive deficiencies not seen in animals uniformly deficient in Btk activity, we sought to evaluate the Btk^- phenotype in chimeric animals. We selected as host blastocysts both normal C57BL/6 (B6) embryos, and those from $RAG2^{-/-}$ mice (Shinkai et al., 1992), which lack a functional gene involved in the initiation of immunoglobulin and T cell receptor gene rearrangements (Schatz et al., 1989; Oettinger et al., 1991) and therefore do not produce any mature lymphocytes. These alternative host strains permitted us to evaluate how Btk^- ES-derived lymphocyte progenitors and their descendants might fare in distinct selective environments.

Injection of ES cells from 8 Btk^- clones into host blastocysts yielded 42 B6 host animals with both coat color and blood chimerism; similar injection into *RAG* hosts yielded 10 chimeras representing three clones. Chimeric analyses were performed using these animals.

Btk^- B Lymphocytes Are Selectively Absent in the Periphery of B6 Chimeric Animals

Expression of the 129-specific lymphoid cell marker Ly9.1 (Mathieson et al., 1980; Lanier et al., 1981) permits the enumeration of ES-derived lymphocytes in B6 hosts (Gross et al., 1995). Figures 2A–2D show a representative set of flow cytometric profiles from spleen cells of an 8-week-old REK2 Btk^- ES-derived B6 chimera and age-matched B6 control, illustrating that chimeric animals have vastly greater ES contribution to the peripheral T cell lineage than to that for B cells. Similarly analyzed were 41 other blood-chimeric animals derived from multiple REK2 and D3J8 ES clones, with the consistent finding that Btk^- ES-derived B cells are virtually absent in peripheral blood and spleen. The maximum percentage of B cell chimerism observed in the blood was 1.8% above background (Figure 2, bottom). Of interest, scoreable B cell chimerism was evident almost exclusively in animals analyzed at 4 weeks of age or less (data not shown). T cell chimerism, on the other hand, was robust, reaching values of 15%–40%, and was stable with time. These T cells exhibited normal expression patterns of CD3, CD4, and CD8 (data not shown).

Thymocyte Development Proceeds Normally in the Absence of Btk

Within the thymi of B6 chimeric animals, Btk^- ES-derived $CD4^-/CD8^-$ cells progress through the 4^+8^+ stage to CD4 and CD8 single-positive cells in a fashion indistinguishable from that of their B6 host counterparts (Figure 3). The representation of Btk^- cells varied somewhat during development, but was, in general, consistent with levels observed in peripheral T cells (see Figure 2; Figure 3). The profiles in Figure 3 are representative of seven 4-week-old B6 chimeras derived from three independent Btk^- ES clones. Overall chimerism in the thymus was lower in three

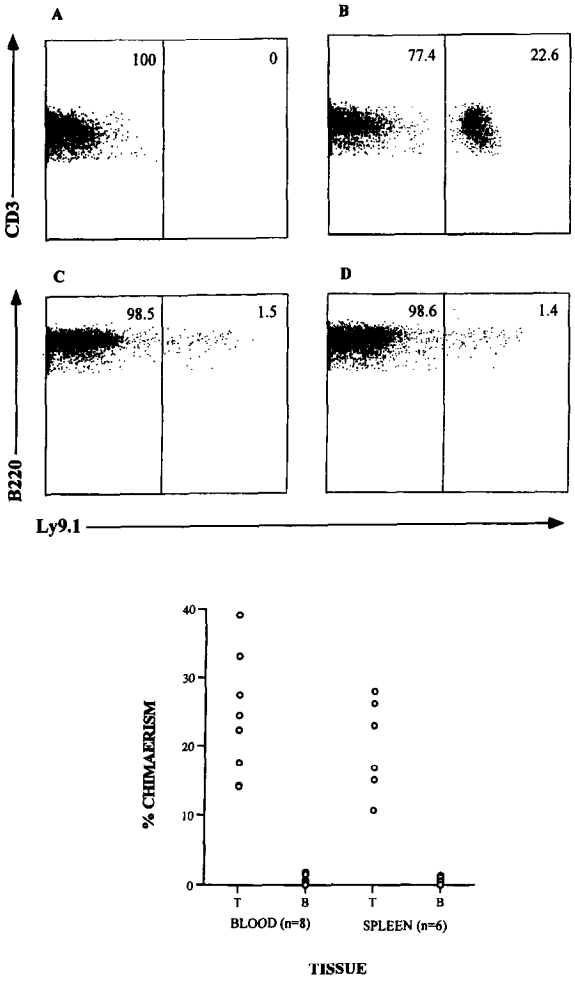

Figure 2. Btk⁻ ES Cells Selectively Yield T Lymphocytes in the Periphery of B6-Host Chimeras

Peripheral blood and spleen samples from C57BL/6 control and C57BL/6/Btk⁻ ES chimeras (denoted as B6 chimeras) were depleted of red blood cells, stained with MAb against CD3, B220, and Ly9.1, and analyzed by flow cytometry. B6 and 129 mice are polymorphic for the lymphoid cell surface antigen Ly9, permitting the tracking of ES-derived (129, Ly9.1⁺) lineages in a wild-type (B6, Ly9.1⁻) host.

(Top) (A) and (B) show Ly9.1 distribution on CD3⁺ gated spleen samples from representative B6 (A), and B6 chimeric animals (B). (C) and (D) show Ly9.1 distribution on the B220⁺ population from the same B6 (C) and B6 chimeric (D) mice. The vertical cutoff shown in each box is derived from similar stains of control 129 splenic lymphocytes. Numbers indicated are percentages of gated events falling into either of the two quadrants.

(Bottom) Summary of peripheral lymphoid chimerism in eight B6 chimeras. Data from analyses including those shown in (A–D) were used to determine the relative percent contribution of Btk⁻ ES cells to peripheral T and B cell populations. Ly9.1⁺ staining percentages from control B6 animals were subtracted from those of chimeric animals, with the resulting Ly9.1⁺ values reflecting true percent chimerism. These values are plotted for peripheral blood stains from eight animals, and for splenic stains from six of the same mice. The data shown are from those animals having the highest levels of peripheral blood chimerism for both B and T lymphocytes. Scoreable peripheral B cell chimerism (never exceeding 1.8% above background) was observed only in animals under 4 weeks of age. The eight animals represent five independent Btk⁻ ES clones. A total of 42 such animals from eight independent Btk⁻ ES clones derived from both REK2 and D3J8 ES cell were analyzed, and those not shown yielded similar results.

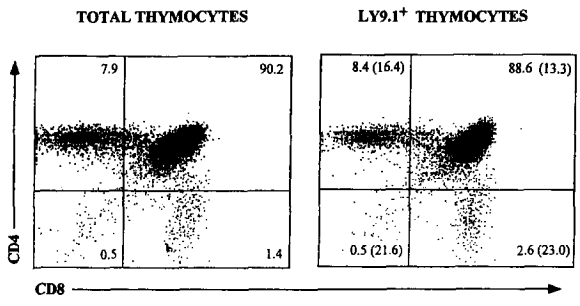

Figure 3. Btk⁻ ES Cells Contribute to Normal Thymocyte Development in B6 Chimeras

Thymocytes from a 4-week-old B6 chimera were stained with MAb against CD4, CD8, and Ly9.1, and analyzed by flow cytometry. The left panel shows total thymocytes (ES- and B6-derived); the right panel shows the Ly9.1⁺ subset within the same thymus. Numbers shown represent percentages in each quadrant; numbers in parentheses indicate percent chimerism within a given quadrant. These profiles are representative of those obtained using seven other animals derived from three independent Btk⁻ REK2 ES clones.

blood-chimeric animals analyzed at 4 months of age, although normal CD4/CD8 distributions were maintained among the ES-derived populations (data not shown).

B Lymphocyte Precursor Expansion Is Compromised in Mutant Bone Marrow Populations

The presence of low, yet measurable, numbers of Btk⁻ ES-derived B cells in the periphery of very young B6 chimeras indicated that B lineage development was occurring, albeit at a reduced rate. We therefore evaluated the progression of ES-derived B lineage development in the bone marrow of these animals. The Ly9.1 marker is expressed on virtually all B220⁺ cells in 129 bone marrow, though at lower levels than on peripheral B cells (data not shown). This allowed us to follow ES-derived B lineage development in five 4-week-old B6 chimeras produced from REK2 Btk⁻ ES clone 5/52 (Figure 4). The expression patterns of CD43, B220, and IgM on B lineage cells have been used to define several B lineage subpopulations (Hardy et al., 1991). A B220⁺/CD43⁺ group includes the earliest detectable B lineages, pro- and pre-B I cells (approximately 2% of total bone marrow), while the B220⁺/CD43⁻ population includes pre-B II and maturing B cells (approximately 20% of marrow). Among B220⁺/CD43⁻ cells, three populations can be defined: B220⁺/IgM⁻ pre-B II cells, B220⁺/IgM⁺ immature B cells, and B220^{bright}/IgM⁺ mature B cells (normally representing on the order of 10%, 6%, and 4% of total marrow, respectively). As these percentages suggest, a dramatic proliferative expansion normally occurs between the pro- and pre-B II stages. Indeed, approximately 20% of pro/pre-B I cells, and 80% of large pre-B II cells (representing 25% of total pre-B II) are actively cycling (Karasuyama et al., 1994; Osmond, 1991).

In B6 chimeric mice, we observed a normal degree of expansion of B6-derived progenitors between the pro-/pre-B I and pre-B II stages (Figure 4, lower left). In contrast, Btk⁻ ES-derived pro-/pre-B I cells, present at levels consis-

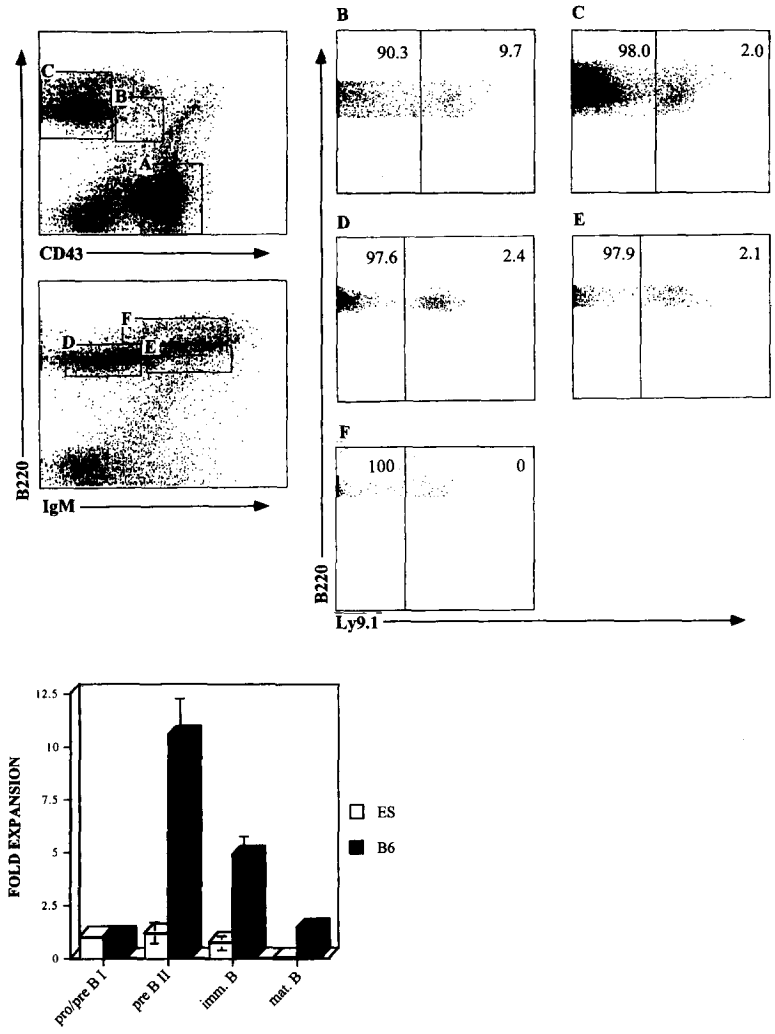

Figure 4. B Lymphocyte Progenitors Fail to Expand Numerically in B6 Chimeric Bone Marrow

(Top) Bone marrow harvested from a 4-week-old B6 chimeric animal was depleted of red blood cells and stained with MAb against B220, CD43, and LY9.1 or B220, IgM, and Ly9.1. Top left, two-parameter stain for B220 and CD43. Middle left, two-parameter stain for B220 and IgM. Boxed areas represent 38% (A), 1.9% (pro- and pre-B I cells; [B]), 26% (pre-B II and maturing B; [C]), 12% (pre-B II; [D]), 7% (immature B; [E]), and 5% (mature B; [F]) of total marrow nucleated cells. B–F show Ly9.1 distribution among the cells from each of these boxed populations. Numbers shown are percentages of Ly9.1$^+$ events, with background staining from B6 controls subtracted. (A) includes Ly9.1$^+$ cells as well, though additional stains demonstrate that these are primarily T lymphocytes (data not shown).

(Bottom) Compilation of data from five 3- to 4-week-old B6 chimeras derived from Btk$^-$ REK2 ES clone 5/52. Total numbers of pro/pre-B I populations (B), separated into Ly9.1$^+$ and Ly9.1$^-$ groups, were each set equal to a value of 1.0. The table plots the expansion of the respective B6- and ES-derived populations into pre-B II (D), immature B (E), and mature B (F) compartments. Note that (D) includes pro-B and pre-B I cells. Hence, the defective expansion of this compartment in Btk$^-$ cells is slightly more profound than the calculation indicates. This method allows standardization of animal data independent of percent chimerism. Values are mean ± SEM. Note a greater than 10-fold deficit (relative to wild type) in the ability of Btk$^-$ B lymphocyte progenitors to expand to or within the pre-B II stage.

tent with overall thymic chimerism for each of the five animals studied, demonstrated a striking inability to yield expected pre-B II cell numbers within the same animals: little if any increase in abundance was observed in all five animals between the ES-derived pro-/pre-B I and pre-B II populations (Figure 4). Beyond the pre-B II stage, B6-derived B cells decreased in abundance as expected (since few of these cells are cycling, and some exit into the periphery), while Btk$^-$ ES-derived B cells declined to undetectable levels by the mature B cell stage. Whether the absence of mature Btk$^-$ ES-derived B cells in these bone marrow samples reflects cell death, early exit from the compartment, or inadequate recirculation from the periphery cannot be ascertained with certainty (see Figure 2).

Peripheral B Cell Numbers Are Partially Rescued in *RAG2*$^{-/-}$ Chimeric Hosts

The relative rarity of ES-derived B cells attaining the periphery of B6 chimeric animals precluded phenotypic analysis of such cells. The RAG *null* blastocyst complementation assay (Chen et al., 1993) has permitted lymphoid development from ES cell clones bearing disruptions of otherwise vital genes, including *csk* (Gross et al., 1995), c-*jun* (Chen et al., 1994), *Rb* (Chen et al., 1993), and *vav* (Tarakhovsky et al., 1995; Zhang et al., 1995; Fischer et al., 1995). Since the *RAG2*$^{-/-}$ block of lymphocyte development is absolute (Shinkai et al., 1992), we evaluated mature B and T cell populations in *RAG2*$^{-/-}$/Btk$^-$ chimeras. We injected *RAG2*$^{-/-}$ blastocysts with ES cells representing three independently derived Btk$^-$ ES clones (two from the D3J8 line and one from REK2). This environment proved more permissive for the development of Btk$^-$ B cells (Figure 5). Peripheral blood and spleen from chimeric animals contained both ES-derived T and B cells. Peripheral blood (Figure 5B) and spleen (Figure 5D) flow cytometric profiles revealed 20%–100% of wild-type T cell abundance, and 1%–15% wild-type B cell abundance. The peripheral B cell percentage was generally 8- to 10-fold less than that of T cells in the animals evaluated (Figure 5B). Both the relative percentages and the abundance of the two cell types remained stable for at least 6 months (data not shown). Of interest, the B cells present in the periphery of *RAG* chimeric animals consistently demonstrated the same IgM^{bright}/IgD^{dull} phenotype characteristic of both *xid* and XLA B cells (Figure 5D).

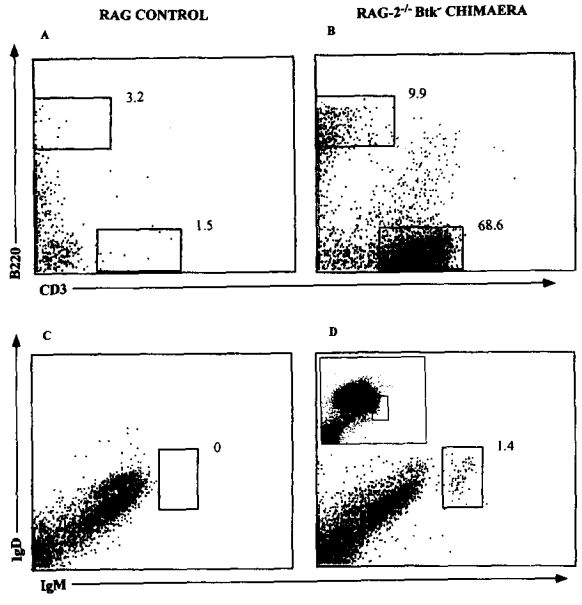

Figure 5. Btk^{-} ES Cells Yield T Cells and Reduced Numbers of B Cells in *RAG2*$^{-/-}$/Btk^{-} ES Chimeras

(A) and (B) Peripheral blood samples from 4-week-old control *RAG2*$^{-/-}$ (A) and *RAG2*$^{-/-}$/Btk^{-} chimeric animals (B) made using Btk^{-} D3J8 ES clone 127 were depleted of red blood cells, stained with MAbs against B220 and CD3, and analyzed via two-color flow cytometry.

(C) and (D) Spleen cells from similar animals were depleted of red cells and stained with MAbs against IgM and IgD. Numbers shown indicate percentages of boxed populations. Note the inset in (D), which shows a 129 splenic IgM/IgD stain; the B lymphocyte box shows the relative position of cells in the *RAG2*$^{-/-}$/Btk^{-} chimera superimposed on this normal tracing. These results are representative of those obtained in analyzing seven animals from two D3J8-derived clones and one REK2-derived Btk^{-} ES clone. B cell abundance in these animals ranged from 1%–15% of wild-type levels, while T cell abundance ranged from 20%–100% that of wild type. Both the B/T cell ratios, and the absolute numbers of these cells, persist over time (data not shown).

Btk^{-} B Cells Respond Selectively to a Thymic-Dependent Antigen

CBA/N mice bearing the *xid* mutation are characteristically nonresponsive to polysaccharide TI-2 antigens (Amsbaugh et al., 1972). Given the cell surface IgM/IgD phenotypic similarity of the Btk^{-} B cells in *RAG2*$^{-/-}$ chimeras (Figure 5D) with those seen in *xid* animals, we wished to extend the comparison to include immune responses. Figure 6 shows the humoral responses of 129, *xid*, *RAG2*$^{-/-}$, and *RAG2*$^{-/-}$/Btk^{-} ES chimeras to antigenic challenge with a thymic-dependent (keyhole limpet hemocyanin, KLH) and a TI-2 antigen (dinitrophenyl [DNP]–Ficoll). As expected, wild-type 129 animals mounted vigorous responses to both antigens, particularly DNP–Ficoll, *xid* animals responded only to KLH, and *RAG2*$^{-/-}$ animals were nonresponsive to both. The *RAG2*$^{-/-}$ chimeric response echoed that seen from the *xid* animals: three of the *RAG2*$^{-/-}$ chimeras produced antibodies in response to KLH immunization, while none mounted any measurable response to DNP–Ficoll. Given that the wild-type (129) response to DNP–Ficoll is of greater magnitude than that to KLH (Figure 6, compare upper and lower left panels), it is unlikely that the failure of the *RAG2*$^{-/-}$ chimeras to respond to the TI-2 antigen merely represents a limiting titration of the two responses. These results demonstrate that Btk^{-} and *xid* B cells have a common defect in the ability to respond to a TI-2 antigen.

Discussion

We have generated somatic cell chimeric animals bearing cell populations with a null mutation in the gene encoding the protein tyrosine kinase Btk. These chimeric animals form an internally controlled set of competition experiments in which both Btk^{-} B and T lymphocyte development can be evaluated against that of nonmutant progenitors. Choosing both wild-type (C57BL/6) and *RAG2*$^{-/-}$ blastocysts as hosts for Btk^{-} ES cells permitted us to control the degree of competition facing Btk^{-} lymphoid progenitors (and their descendants). We studied the ability of Btk^{-} T and B cells to colonize the periphery of both sets of animals, and noted a selective reduction in Btk^{-} B lymphocyte representation. Failure of expansion of early B lineage progenitors in the bone marrow of wild-type host chimeras appears to explain the low numbers of mutant B cells detected in the periphery of these animals.

T Cell Development Proceeds Normally in the Absence of Btk

In B6 host chimeric animals, Btk^{-} ES-derived T cells compete well with their wild-type counterparts, contributing to as much as 40% of peripheral T cell abundance in some animals. Moreover, intrathymic development appeared virtually indistinguishable from that of B6 T cells. In *RAG2*$^{-/-}$ hosts, normal T cell abundance was typically observed. In addition, the ability of Btk^{-} B lymphocytes to respond to KLH documents that mutant T cells are able to deliver specific help towards antibody production. The finding of normal T cell development and function in the absence of Btk is not unexpected, since there is no consistent evidence from either XLA patients or *xid* animals for any intrinsic defect in the T cell lineage (Richards et al., 1992; Crockard et al., 1992). Nonetheless, the presence of T cell chimerism proved a useful index for comparison with mutant B cell levels, and also formally demonstrates that an absence of mutant B cells could not be attributed to loss of any common lymphocytic precursor.

Btk^{-} B Cells Display an *xid* Phenotype in the Periphery of Chimeric Hosts

In contrast with the abundance of Btk^{-} ES-derived T cells in the periphery of chimeric animals, virtually no mutant B cells were detected in either peripheral blood or spleen from most chimeras. An exception appears to be young mice: chimerism approaching 2% has been detected in animals assayed at under 4 weeks of age. This finding is consistent with previously published observations regarding the *xid* phenotype. The peripheral B cell population in female mice heterozygous for the *xid* mutation (*xid*/+) exhibits marked skewing of X inactivation in favor of the wild-type X chromosome, and this skewing progresses over time, becoming nearly absolute in mice over 3 months

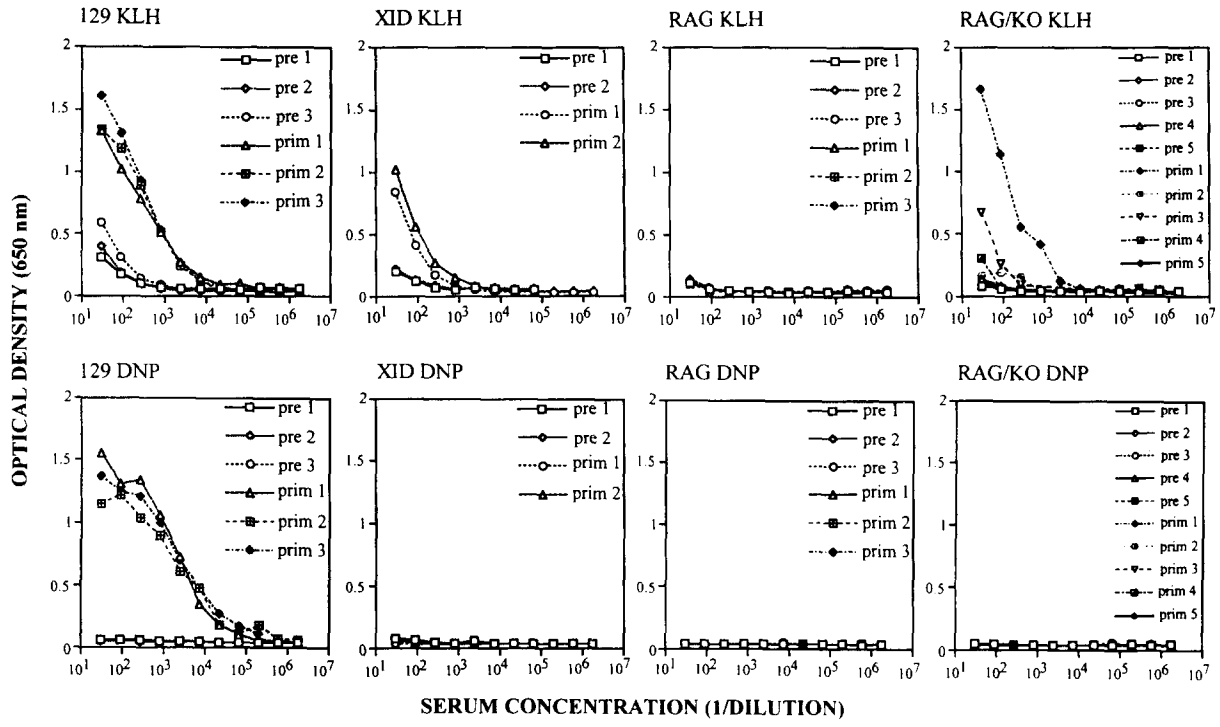

Figure 6. Antibody Production by Btk⁻ ES-derived B Cells

We prebled and immunized three 129, two CBA/N*xid*, three *RAG* control, and five *RAG* chimeras from D3J8 Btk⁻ ES clone 127 2 months postweaning with 10 μg of DNP–Ficoll. The animals were bled 8 days later and immunized with 100 μg of KLH in a 1:1 mixture with complete Freund's adjuvant. A final bleed was performed after an additional 10 days. KLH and DNP IgM responses were measured via ELISAs in serial dilution as described in Experimental Procedures.

of age (Forrester et al., 1987; Nahm et al., 1983). In irradiated hosts reconstituted with a mixture of normal and *xid* stem cells, mutant B cells differentiate but are eventually completely displaced in the periphery, presumably by more efficient expansion of wild-type cells (Sprent and Bruce, 1984). In concert with these findings, rare mutant B cells in our B6 chimeras disappeared over time, while B cell numbers in the periphery of *RAG2*$^{-/-}$ chimeras, where competition could not occur, remained stable.

An immature IgM/IgD cell surface ratio and the selective inability to respond to TI-2 antigens are hallmarks of *xid* B cells (Wicker and Scher, 1986). The B cells observed in the blood and spleens of our *RAG2*$^{-/-}$/Btk⁻ chimeras exhibit these same cell surface and immune response characteristics. The abundance of peripheral B cell numbers in *xid* animals is approximately one-third that of normal animals (Sprent et al., 1985). In *RAG2*$^{-/-}$/Btk⁻ chimeras, we have not observed B cell abundance over 15% that of wild type. There are several possible explanations for the reduced numbers of B cells in these chimeras. Competition in the periphery cannot readily explain the difference in B cell abundance seen between our chimeras and *xid* animals, given the complete lack of *RAG2*$^{-/-}$-derived lymphocytes in such hosts. The necessity for T cell help in the absence of full Btk function is indicated by the observation that *xid* × *nu* (athymic) mice, and thymectomized irradiated recipients of T cell–depleted *xid* bone marrow demonstrate a severe deficit of mature B cells (Wortis et al., 1982; Mond et al., 1982; Sprent and Bruce, 1984). Given this T cell dependence, the relative diminution in B cell numbers in the *RAG2*$^{-/-}$ chimeras as compared with *xid* mice might be explained in part by subnormal T cell numbers. However, this explanation is unlikely, given that the B:T ratios in *RAG* chimeras remain essentially constant over the 20%–100% of wild-type range of T cell abundance observed in the same animals. Most likely, much of the difference can be attributed to competition in the bone marrow between Btk⁻ progenitors and early host *RAG2*$^{-/-}$ progenitors (Karasuyama et al., 1994; Melchers et al., 1994). In addition, background gene variation between the ES (129)/host (mixed 129 × B6) strain combination and the original *xid*-bearing CBA/N strain may contribute to the difference. While the ability of *xid* B cell progenitors to mature in *RAG2*$^{-/-}$ hosts has not been tested, our results from splenic and blood lymphoid studies in both B6 and *RAG* host chimeras suggest that there exist no profound differences between Btk⁻ and *xid* peripheral B cells.

A Competitive Disability in the Expansion of Pre-B II Cells Distinguishes Btk⁻ from *xid* Lymphoid Progenitors

In the bone marrow of normal animals, a large numeric expansion (10-fold or more) occurs between the pro- and pre-B II stages of B cell development (Melchers et al., 1994), mainly as a consequence of the high percentage of large pre-B II cells that are actively cycling (Karasuyama et al., 1994; Osmond, 1991). These cells initially express both cytoplasmic IgM heavy chains (cμH) and the $V_{PRE\text{-}B}$/

λ5 surrogate light chain (SL) (Cherayil and Pillai, 1991), and both are necessary for expansion in the pre-B II stage (Karasuyama et al., 1994). Given the relative lack of Btk⁻ ES-derived B cells in the periphery of our B6 chimeric animals, we sought to track the development of both B6- and Btk⁻ ES-derived B cell progenitors in an attempt to define point(s) along the pathway at which Btk might normally exert its effects. By scoring the relative numbers of B6- and ES-derived precursors passing from the pro-/pre-B I through mature B cell stages, we observed a greater than 10-fold deficit in the ability of mutant progenitors to expand between the pro-/pre-B I and pre-B II stages. In principle, this failure could be attributed to either inadequate clonal expansion or excessive cell death at or immediately prior to the pre-B II cell stage. Since in the *RAG2*$^{-/-}$ host chimeras (where B cell development is blocked prior to the pre-B II cell stage) B cell numbers are both significantly restored and stable over time, an intrinsically increased rate of Btk⁻ progenitor cell death at the pre-B II stage almost certainly does not explain the poor developmental capacity of these progenitors in B6 hosts. This finding suggests, therefore, that the attrition of maturing Btk⁻ B cells observed in B6 chimeric bone marrow results from a competitive process.

xid animals have relatively normal numbers of pro-B, pre-B, and maturing B cells in their bone marrow (Kincade et al., 1982). Moreover, the proliferative rate of cytoplasmic μ⁺ pre-B cells and the production of small lymphocytes proceeds normally (Reid and Osmond, 1985). In *xid*/+ animals, selection against *xid* cells in bone marrow is evident for surface IgM⁺ B cells, but not for B220⁺/IgM⁻ pre-B cells (Forrester et al., 1987). The lack of selection against *xid* pre-B cells clearly distinguishes the *xid* phenotype from that of a true murine Btk⁻ mutation as defined here.

In humans, there is no skewing of X inactivation among CD19⁺/CD34⁺ early pre-B cells in female XLA carriers (Conley et al., 1994). But the ability of XLA patients to produce pre-B II cells is variable, and a generalized proliferative defect at this stage has been described (Campana et al., 1990). Our findings demonstrate that the Btk⁻ B-cell lineage in the mouse manifests a similar abnormality in precursor cell expansion. Viewing these results in aggregate, the phenotypic heterogeneity resulting from loss of Btk function appears to be largely quantitative: in both mouse and human there is a competitive, but not absolute, compromise in B progenitor cell expansion.

The Consequences of Btk Dysfunction: Reconciling Mouse and Man

At least two patients with XLA have been shown to bear a *BTK* mutation in precisely the same codon as that affected in the *xid* mouse (de Weers et al., 1994a; Ohta et al., 1994), though the mutation is different (R28H, as opposed to the murine R28C). This observation suggests that mice and humans may be at least partially discordant regarding the necessity of Btk. However, meaningful comparison is difficult: a high degree of clinical heterogeneity exists among XLA patients, and as more of the mutations involved in individual cases have been catalogued (Kornfeld et al., 1994; Duriez et al., 1994; Bradley et al., 1994; Conley et al., 1994), it is becoming increasingly apparent that there may be little (if any) correlation between the type of mutation present and the severity of disease in any given patient. Bone marrow– or T cell–specific background genes, as well as the age of a patient and prior infection history, are likely to influence the severity of the disease. Many XLA patients appear to have low but measurable numbers of circulating B cells, and these cells exhibit the same IgM^{bright}/IgD^{dull} phenotype characteristic of both *xid* (Conley, 1985) and murine Btk⁻ B cells. Given the similarities in mice and humans between circulating Btk⁻ B cells, and the common defect in expansion to or within the pre-B II cell stage of B lineage development, we feel that Btk deficiency disease in both species yields an identical abnormality in B lymphopoiesis, with variable severity in humans, owing to the existence of both genetic and environmental modifying factors. XLA patients are, to an appreciable extent, self-selecting, and hence there is a considerable bias of ascertainment. To our knowledge, there is no catalogue of humans bearing *Btk* mutations who are not sick, though such potentially *xid*-like individuals may exist.

What does Btk actually do? The kinase has been shown to be activated in response to FcεRI cross-linking in mast cells (Kawakami et al., 1994), and IL-5 treatment (Appleby et al., 1995) or ligation of surface immunoglobulin (Aoki, 1994; Saouaf et al., 1994; de Weers et al., 1994b) in B cells. In addition, in a pre-B cell line it is constitutively phosphorylated on tyrosine residues (Aoki, 1994). Given a potential role in signal transduction, we speculate that Btk may not only be involved in the signaling activity of relatively mature cells, but also in the transduction of signals, perhaps mediated by the pre-B cell receptor, regulating expansion to or within the pre-B II stage of B-cell development. The R28C mutation, associated with the *xid* phenotype, has been shown to perturb an interaction between the kinase and several PKC isoforms (Yao et al., 1994). Our results suggest this mutation may affect the various signaling functions of murine Btk differentially. However, the close similarity between phenotypes resulting from this mutation and the fully disabling mutation that we have engineered argues that PH domain function is indispensable for Btk-mediated signal transduction, and almost certainly for the normal function of other proteins containing such structures.

Experimental Procedures

Construction of BTKN.1 Targeting Vector and Homologous Recombination in ES Cells

A disruption vector was created using a 9 kb BamHI subclone from a 20 kb *btk* unique region mouse genomic clone (Figure 1A, top) (Rawlings et al., 1993) isolated from a 129Sv I genomic library (provided by P. Soriano). A 3.5 kb EcoRI–SpeI fragment (containing coding exons 3 and 4) was replaced with the pMC1polA neomycin phosphotransferase cassette (in inverse orientation), and the resulting sequence cloned into the herpes simplex virus thymidylate kinase (*tk*) gene in pKS. When linearized, the vector contains 1.5 kb of 5′ sequence homology and 4 kb of 3′ homology adjoining the *tk* cassette (Figure 1A, middle). The vector was electroporated into 1 × 10^8 D3J8 (gift of R. Jaenisch) or REK2 (Brandon et al., 1995; gift of S. McKnight) ES cells on three

occasions, with conditions and G418/gancyclovir selection performed as described (Appleby et al., 1992), with the exception that REK2 cells were maintained on primary mouse embryonic fibroblast monolayers rather than on the STO fibroblast line. Clones were screened for homologous integrations via genomic Southern blotting of ES DNA with a 5′ *btk* cDNA probe (coding exons 1–5), and candidates evaluated with additional probing strategies (Figure 1B). All clones used for analysis in chimeric animals were from either different electroporations or separate platings within a given electroporation.

Blastocyst Injections and Transfers

ES cells (15–20) from individual clones were injected into 3.5-day-old embryos harvested from C57BL/6 or *RAG2*$^{-/-}$ mice (mixed 129/Sv and Bl/6 background, breeders for which were provided by F. W. Alt) and transferred into sham-mated Swiss Webster recipients as described (Robertson, 1987). B6 chimeras were housed in both specific pathogen-free (SPF) and conventional facilities. *RAG2*$^{-/-}$ chimeras were housed only under SPF conditions.

Flow Cytometry

Lymphocyte suspensions were prepared from whole organs or peripheral blood, with spleen, bone marrow, and peripheral blood samples depleted of red blood cells using ammonium chloride as described (Cooke et al., 1991). Monoclonal antibodies (MAbs) conjugated to fluorescein isothiocyanate, phycoerythrin, or biotin and specific for murine B220 (Caltag Labs, San Francisco, California) CD43, CD4, CD8, CD3ε, Ly9.1 (PharMingen, San Diego, California) and IgD (The Binding Site, San Diego, California), plus tricolor-conjugated streptavidin (Caltag Labs) were used to stain 1 × 10^6 cells as described (Gross et al., 1995), with events collected in list mode files on a FACScan flow cytometer (Becton Dickinson) with Lysis II software, and analyzed with Reproman Software version 2.07 (Truefacts Software, Seattle, Washington).

B Cell Isolation and Sequence Analysis of *btk* Transcripts

Splenocytes (2 × 10^7) from a 129 control mouse and a *RAG2*$^{-/-}$/Btk$^-$ chimeric animal were stained with MAbs specific for CD3 (fluorescein isothiocyanate–conjugated) and B220 (phycoerythrin-conjugated) as described above. B220$^+$/CD3$^-$ cells (1 × 10^5) with lymphoid forward/side scatter characteristics were sorted on a dual laser FACStar Plus instrument (Becton Dickinson Immunocytochemistry Systems, San Jose, California) and frozen. Total nucleic acids were prepared as described (Gross et al., 1995) and used for RT–PCR with protocols and materials from GIBCO BRL (Gaithersburg, Maryland) and Perkin–Elmer Cetus (Norwalk, Connecticut). Gel electrophoresis, hybridization, and probing were accomplished with standard procedures. *btk* primer sequences (defined based on the known *btk* genomic structure) (Sideras et al., 1994) were the following: exon 1, 5′-ATGGCTGCAGTG ATACTGGAGAGC-3′; exon 2, 5′-AGAAGAGGCAGTAAGAAAGGT-TCA-3′; exon 2*, 5′-GAATCTGTCTTTCTGGTGGGGGAT-3′; exon 3, 5′-AGGAGAGGTGAGGAGTCTAGTGAA-3′; exon 4, 5′-GTTGTATAT-GATGAAGGACCTCTC-3′; exon 5/6, 5′-CCAGGTTTTAAGCTTCCA-TTCCTG-3′; exon 6, 5′-GTAGGGGGAAGAGGCTTTTTCGTT-3′; exon 12/13, 5′-TTTTCCCATGATCCATAGCCCAGGC-3′; and exon 13, 5′-AGCTCCTTCAAGAAGGTGAGGTCC-3′. Mutant and wild-type products amplified with the exon 1 and exon 5/6 primer pair were excised from ethidium bromide–stained agarose gels, digested with PstI and HindIII (which cut uniquely within primers 1 and 5/6, respectively), and ligated into pKS (Stratagene, La Jolla, California). Sequencing was performed according to the chain termination method (Sanger et al., 1977) using Sequenase Version 2.0 (United States Biochemicals, Cleveland, Ohio).

Serology

Animals were pre-bled (200 μl) and immunized 2 months postweaning with 10 μg of DNP–Ficoll in phosphate-buffered saline (provided by S. Bondada) intraperitoneally on day 0. Mice were bled on day 8, immunized intraperitoneally with 100 μg of KLH (Sigma Chemical Company, Saint Louis, Missouri) in a 1:1 homogenate with complete Freund's adjuvant (Life Technologies, Incorporated, Grand Island, New York), and bled once more on day 18.

Anti-DNP immunoglobulin isotype levels were determined with a modified sandwich enzyme-linked immunosorbent assay (ELISA) as described (Renshaw et al., 1994). In brief, individual wells of 96-well plates (Flow Laboratories, Incorporated, McLean, Virginia) were coated with anti-mouse IgM antibody (Southern Biotechnology Association, Birmingham, Alabama) and blocked with 5% nonfat dry milk in phosphate-buffered saline. Plates were then incubated with serially diluted test serum samples. Biotinylated TNP–bovine γ globulin (TNP–BGG; cross-reacts with anti-DNP antibodies) was used as a third step followed by horseradish peroxidase–conjugated streptavidin (Zymed, South San Francisco, California). All assays were developed using the TMB Microwell peroxidase substrate system (Kierkegaard and Perry Laboratories, Incorporated, Gaithersburg, Maryland). Plates were then read on a Dynatech ELISA reader (Dynatech Labs, Incorporated, Chantilly, Virginia).

Anti-KLH IgM levels were determined by a direct ELISA method. In brief, wells were coated with 1 mg/ml KLH in phosphate-buffered saline and blocked with 5% nonfat dry milk. Serially diluted serum samples were added, followed by horseradish peroxidase–conjugated goat anti-mouse IgM (Southern Biotechnology Association). Plates were developed with peroxidase substrate and analyzed as described above.

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